
بسم الله الرحمن الرحیم
IN THE NAME OF GOD
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ANTITUMOR ACTIVITY OF NANOLIPOSOMES ENCAPSULATING THE NOVOBIOCIN ANALOG 6BRCAQ IN A TRIPLE-NEGATIVE BREAST CANCER MODEL IN MICE

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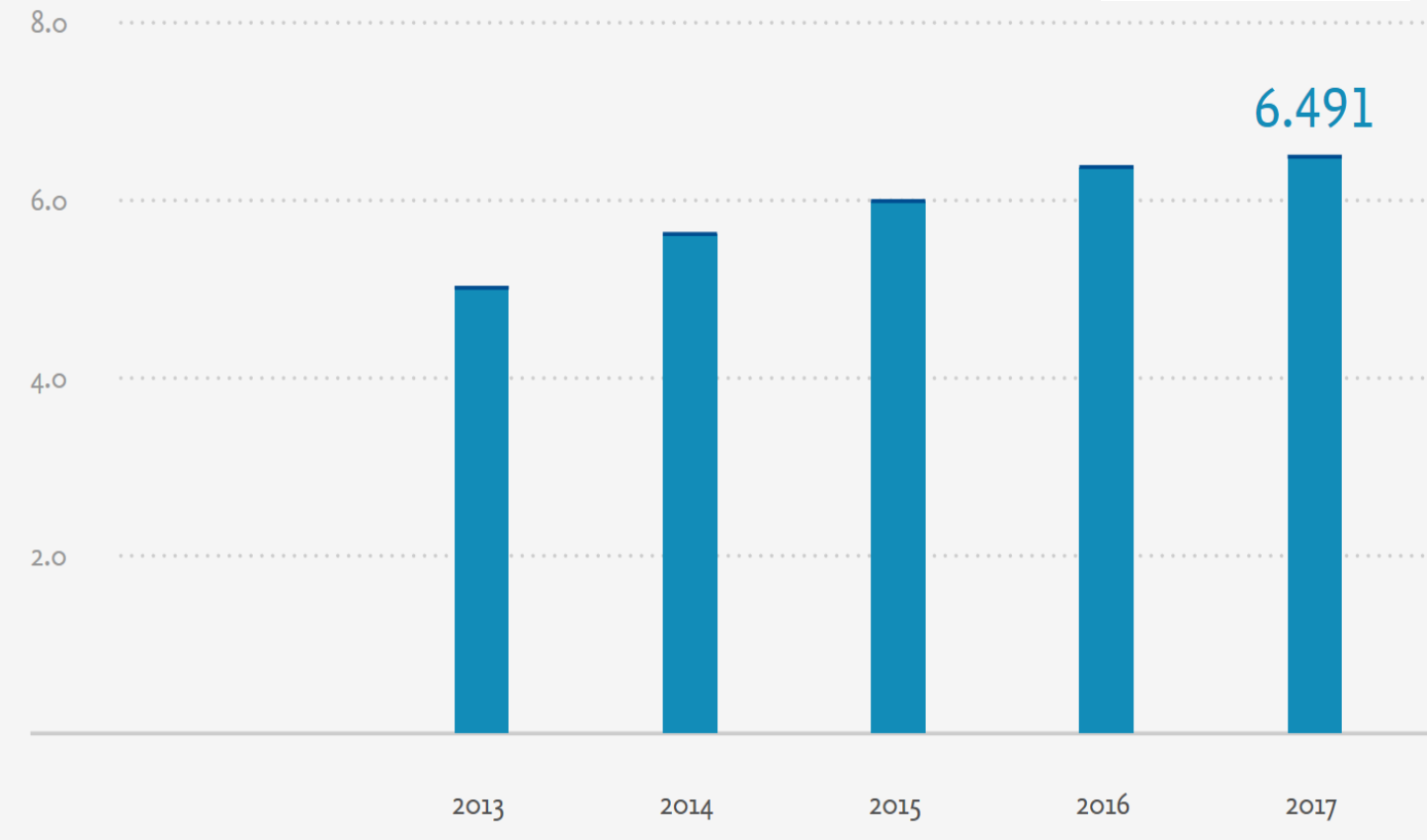


➢ Introduction:

- Hsp90 (Heat shock protein 90) a well-conserved protein
- Involved in the proper refolding of proteins
- located in the cytosol as Hsp90α and β
- Possesses isoforms in mitochondria (hsp75,TRAP-1)
- endoplasmic reticulum (grp94)
- can repaire Almost 400 proteins ⟹ some of which facilitating the oncogenic process
- Targeting hsp90 ⟹ promising strategy
- Hsp90 is part of a complex machinery
- works together with hsp70, hsp40

- consists of three domains:
- N-terminal domain involved in ATP hydrolysis
- intermediate domain with high affinity for client proteins
- C-terminal domain responsible for dimerization
- N-terminal inhibitors such as geldanamycin ⟹ promising results in solid tumors
- side effects and poor tolerance to the treatment
- induce an overexpression of hsp70 ⟹ HSR

- constant efforts to synthesize more efficient compounds
- higher clinical tolerability and that do not induceca HSR
- Among the nvb derivatives ⇒ 6BrCaQ
- promising results in MCF-7 cells
- anti-proliferative effects in vitro
- client protein degradation
- very insoluble in aqueous media
- Our strategy ⇒ use nanoparticulate carriers
- overcome solubility problems and improve bioavailability
- also to specifically deliver them

- In a previous study ⇒ encapsulated 6BrCaQ in PEGylated liposomes in the PC-3 prostate cancer cell line
- pro-apoptotic effects, a G2/M cell cycle blockade and a synergistic effect with doxorubicin
- liposomal 6BrCaQ retarded cell migration
- PEGylated liposomes can take advantage ⇒ enhanced permeation and retention effect (EPR)
- higher permeability of tumor blood vessels
- reduced lymphatic drainage
- resulting in a better accumulation at the cancer site

➢ Material and methods

Chemicals:

- 6BrCaQ

The pure compound was solubilized at 10mM in chloroform for liposome preparation and in DMSO for use as a free drug.

- Egg chicken L-α- phosphatidylcholine (Egg PC)
- Cholesterol (CH)
- DSPE-PEG2000
- Rho-PE

Stock solutions of lipids (25 mg/ml for each) were made in chloroform.

Cell lines and culture:

- MDA-MB-231 cells, either wild type or expressing GFP and firefly luciferase (Luc2-GFP)
- negative for Mycoplasma ⟹ Mycoalert™ detection kit
- MDA-MB-231 wild type ⟹ L15 medium supplemented with 15% heat-inactivated fetal bovine serum, 2mM glutamine, 22mM NaHCO3 and 0.5% of Penicillin-Streptomycin
- MDA-MB-231 Luc2-GFP ⟹ EMEM supplemented with 10% heat-inactivated fetal bovine serum, 2mM glutamine and 0.5% of Penicillin-Streptomycin
- were incubated in a humidified atmosphere containing 5% CO2 at 37 °C

Liposome preparation:

- composed of Egg PC, Cholesterol and DSPE-PEG2000 (2.17:1:0.17 respectively molar ratio)
- were formed ⟹ by the thin film hydration method
- mixture was dried ⟹ at 25 °C using a rotary evaporator for 60 min
- film was hydrated a sterile-filtered buffer solution
- (10mM HEPES,145mM NaCl, pH=7.4) at room temperature
- The multilamellar liposomes formed were sequentially extruded through polycarbonate membranes

- 6BrCaQ was added in excess ⟹ an optimal drug-to-lipid ratio could be reached
- The extrusion process was able to remove most of the non-incorporated 6BrCaQ, which remained on the membrane after extrusion
- Rhodamine-PE (1% mol) replacing 1% mol of Egg PC in chloroform mixture ⟹ For in-vitro uptake experiments

Determination of encapsulation efficiency and drug-to-lipid ratio in the liposomes:

- to remove non-encapsulated drug ⟹ low-speed centrifugation (10 min; 10,000 g)
- to determine the liposome-associated drug in the supernatant
- ⟹ fluorescence assay (λexcitation=340 nm; λemission=390 nm)
- Lipids did not interfere with the fluorescence of 6BrCaQ at the wavelengths used
- To assess lipid recovery, the PC content was assayed ⟹ an enzymatic colorimetric kit

- calculation the total lipid concentration
- assuming that the proportions remained constant
- Encapsulation efficiency (EE%)
- drug loading (D/L%)

$$EE\ (\%) = \frac{[6BrCaQ]supernatant\ after\ centrifugation}{[6BrCaQ]before\ centrifugation} * 100$$

$$\frac{D}{L}(\%) = \frac{[6BrCaQ]supernatant\ after\ centrifugation}{[Lipids]supernatant\ after\ centrifugation} * 100$$

Size and zeta potential measurements:

- The mean hydrodynamic diameter of the liposomes ⟹
  Determined by Dynamic Light Scattering
- Samples were diluted in pure water prior to the measurements
- analysed at a backscatter angle of 173°
- Polydispersity index (PDI) was used as an indicator of size distribution.
- Zeta potential was measured in a folded capillary cell after 30-fold dilution in pure water

Cell cycle analysis:

- MDA-MB-231 cells were seeded for 24 h in a 6-well plate (10^5/well)
- treated with 10 μM of liposomal 6BrCaQ or empty liposomes for 24 h
- Thereafter cells were washed in PBS, recovered in a sufficient number (2×10^5 cells) and permeabilized in ethanol 70% (−20 °C)
- On the day of analysis ⟹ centrifugation
- staining by a PBS solution containing DNAse free ribonuclease A (100 μg/mL) and propidium iodide (PI) (12.5 μg/mL)
- Data were then acquired with a flow cytometer and the
- cell cycle was analysed ⟹ FlowJo software

Cell viability determination (MTS assay and trypan blue staining):

- Cell viability was determined ⟹ the Cell Proliferation Assay (Promega)
- MDA-MB-231 (5000 cells/well) were seeded into 96-well plates
- treated with loaded liposomes, free drug and vehicle controls for 24 h
- Each culture condition was analysed in 5 wells for each plate and the experiment was repeated three times.
- Absorbance values at 492 nm

- The absorbance recorded with non-treated cells was taken as 100% of metabolic activity corresponding to maximum viability.
- Cell viability and proliferation were determined after 24 h treatment by Trypan blue exclusion
- Each sample was diluted with Trypan blue ⟹ 1:1 ratio
- Viable and dead cells were then counted by optical microscopy

Real-time cell analysis (xCELLigence technology):

- The biological activity of liposomes containing 6BrCaQ was assessed ⇒
- with the Real-Time Cell Analyzer multi-plate (RTCA MP) Instrument
- using the xCELLigence System
- 5×10^{3} MDA-MB-231 cells/per well ⇒ were plated in an E-Plate View 96
- placed in the RTCA MP located inside a cell culture incubator
- left to grow for 24 h before treatment
- impedance was continuously measured until the end of the treatment
- Standard deviations of well replicates were analysed with the RTCA Software

Confocal microscopy:

- Cells were seeded in Lab-Tek 8-well plates
- After 24 h, culture medium was replaced ➡
- medium containing liposomes incorporating a rhodamine-labelled phospholipid (1% molar ratio)
- cells were incubated up to 4 h with liposomes at 37 °C (1.6×10^4 cells)
- then washing with cold PBS
- followed by fixation in paraformaldehyde (2%)
- The association of liposomes with the cells was observed by confocal microscopy

http://microscopy.wfu.edu/instrumentation/confocal-microscope/

Western blots:

- Frozen cell pellets were lysed in RIPA buffer containing phosphatase and protease inhibitors for 30 min on ice
- Protein concentrations in lysates were assayed using Biorad protein assay (Bradford method)
- SDS-PAGE (4–15% or 4–20% acrylamide to obtain a good resolution of the protein profile) (150–200 V for about one hour)
- Proteins were transferred to a PVDF membrane (100 V 45 min) (liquid transfer)
- the quality of the transfer was checked by Ponceau Red staining.

- For immunodetection ⇒ membranes were saturated in 5% non-fat milk TBS- Tween 0.1% for one hour at room temperature
- incubated with primary antibody dilutions overnight (4 °C)
- anti-Hsp70, anti-Hsp90α/β, anti-CDK-4, anti-PARP-1, and anti-GAPDH
- After washing, horse-radish peroxidasecoupled secondary antibodies (anti-mouse) were added for 1 h
- Chemiluminescent signals were analysed using the MF ChemiBis system
- densitometry analysis was performed using MultiGauge Software.

Real time RT-PCR:

- At the end of the treatment ⇨ cells are collected and pellets
- Were washed twice with sterile PBS and kept dry at −80 °C freezer
- Total cellular RNA ⇨ was isolated using RNeasy Mini Kit columns
- RNA quantification ⇨ measuring the absorbance at 260 nm
- 2 μL of each sample are used to measure
- absorbances at 230–260 – 270–280 and 320

- Samples of a RIN index lower than 8.0 were discarded
- 400 ng aliquot of total RNA ⇨ was retrotranscribed using the iScript™ cDNA Synthesis Kit
- After dilution, 3 ng of cDNA was then subjected to real-time PCR analysis
- using the SYBR Green
- following specific primers systems
- Specific amplification of the gene of interest was checked by analysis of melting curves

In-vivo orthotopic breast cancer model:

- on 4-6 weeks-old female nude mice (Fox1nu,nu/nu)
- Cultured MDA-MB-231-Luc2-GFP cells
- recovered using trypsin, centrifuged (300 g; 5 min) and washed three times in PBS
- Cells were then injected to the mammary fat pad of mice (500,000 cells in 50 μL of PBS) one week after animal arrival
- Mice were then checked every day for recovery
- No differences in terms of weight were observed between mice receiving surgery and untreated mice

- Bioluminescence was monitored weekly
- D-luciferin (150 mg/kg) was injected intraperitoneally
- Bioluminescence (photons/sec) was then acquired 6 min after injection
- Obtained images were analysed ⇒ the Living Image Software
- Initiatation of treatment ⇒ tumors reached a size of 60mm3 3–4 weeks after tumor implantation
- liposomes injected into the tail vein once a week until the end of the experiment (34 days after treatment Initiation)

Histology:

- Tumors were recovered from mice on euthanasia 34 days after the beginning of treatment
- mice had received 5 injections
- fixed in a 4% formaldehyde solution overnight at 4 °C
- Then, washing three times in PBS
- preserving in ethanol 70 °C before inclusion in paraffin
- hematoxylin and eosin Staining
- Slides were scanned and analysed with Imagescope software

Immunohistochemical analysis:

- using a Ventana Discovery XT autostainer on 4 μm-thick paraffin sections
- Slides deparaffinizing with EZPrep buffer
- epitopes were unmasked by 15 min high-temperature treatment in CC1 EDTA buffer
- Sections were incubated 40 min at room temperature with anti-Ki67 antibody or cleaved caspase 3 antibody
- Secondary antibody was incubated 16 min at room temperature
- After washes, staining was performed with DAB
- sections were counterstained with Hematoxylin using Ventana reagents

Image analysis:

- The proportion of necrotic area ⟹ Aperio Imagescope software
- Analyses of proliferation ⟹ Ki67 staining
- apoptosis ⟹ cleaved-caspase 3 staining
- using 6 randomly chosen fields selected on each histological section
- Ki67 and cleaved-caspase 3-positive cells countting ⟹ Image J software

Statistical significance:

- All data were expressed as mean ± SEM
- checking Gaussian distribution
- differences between experimental groups ➡
- ANOVA and student's t-test
- Data were considered statistically significant : $p < 0.05$
- Two independent experiments
- the results were pooled
- After checking Gaussian distribution, aberrant values were removed

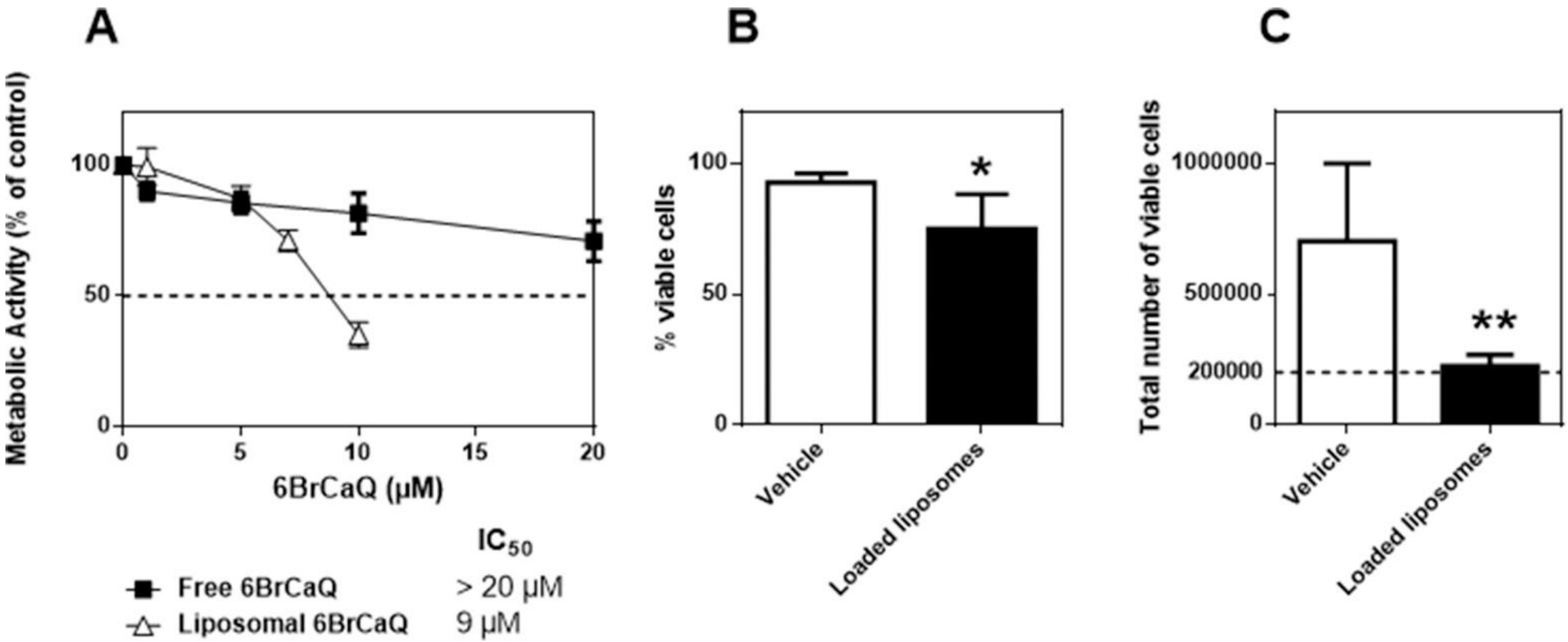
➢ Results

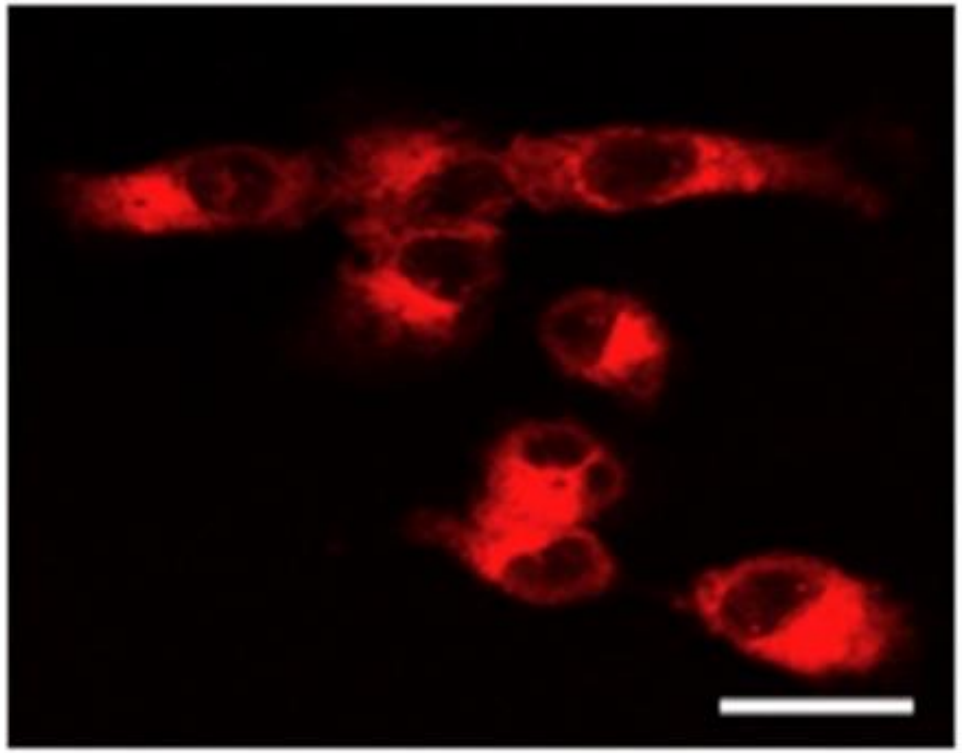
Characterization of liposomes:

- Empty liposomes ⟹ a mean diameter of 124 nm
- loaded liposomes ⟹ a similar diameter of around 121 nm
- Both preparations had a PDI<0.2 ⟹ monodisperse distributions
- Both showed similar surface charge (both around −13 mV)

Anti-proliferative and cytotoxic effects in vitro:

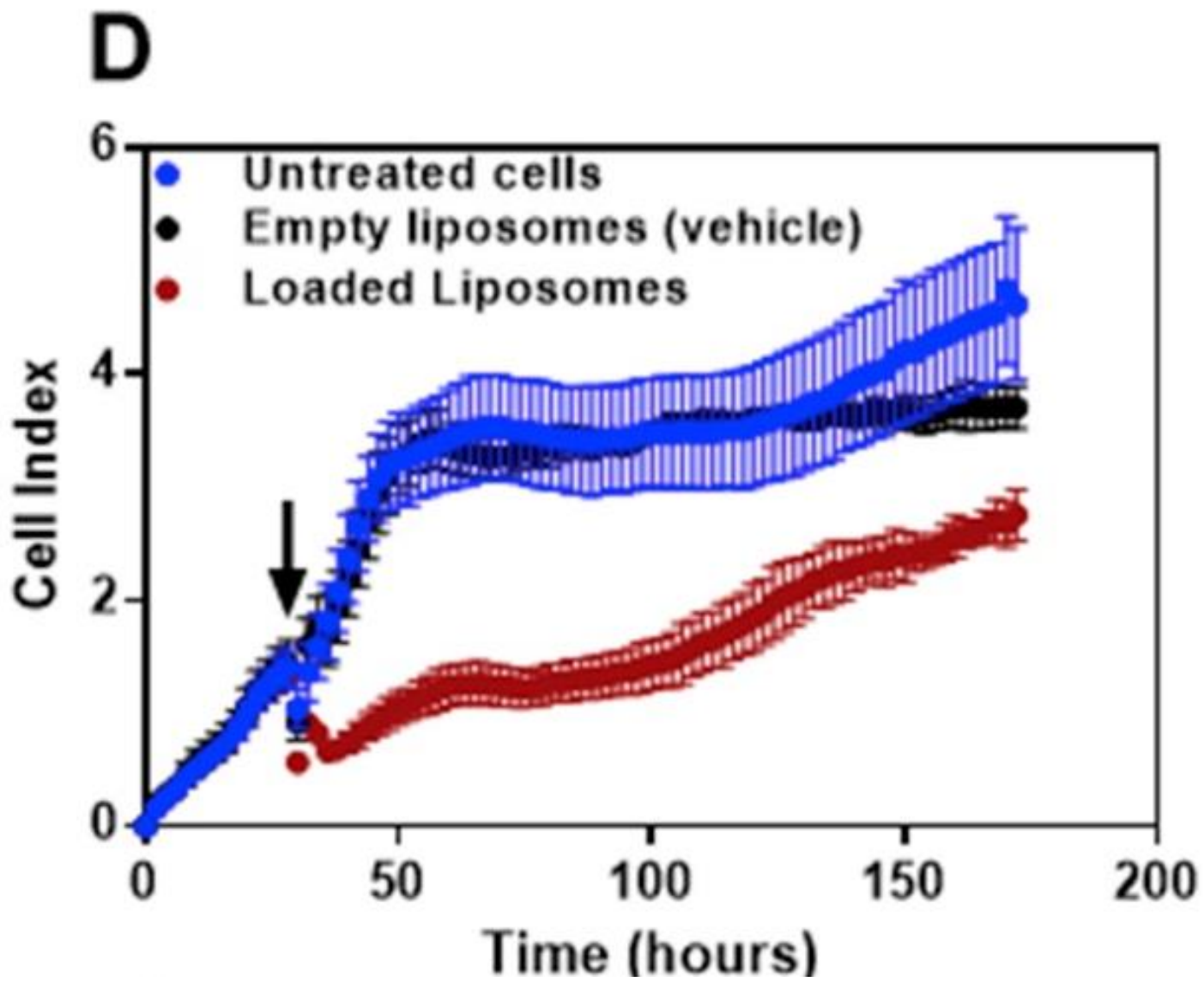
- rhodamine-labelled liposomes ⇒ could be seen within the cells after 4 h
- The metabolic activity of MDA-MB-231 cells:
- around 35% for liposomal 6BrCaQ against 81% for the free drug with MTS assay
- checking the effect on proliferation ⇒ trypan blue staining,after 24 h:
- A slight but significant effect on cell viability between loaded and empty liposomes (92% and 75% respectively)
- a more significant and important effect on the number of cells remaining after a treatment of 24 h (7×10^5 for empty liposomes against 2×10^5 for loaded liposomes)


A
Metabolic Activity (% of control)
100
50
0
0
5
10
15
20
6BrCaQ (μM)
IC50
Free 6BrCaQ
> 20 μM
Liposomal 6BrCaQ
9 μM
B
% viable cells
100
50
0
*
Vehicle
Loaded liposomes
C
Total number of viable cells
1000000
500000
200000
0
**
Vehicle
Loaded liposomes

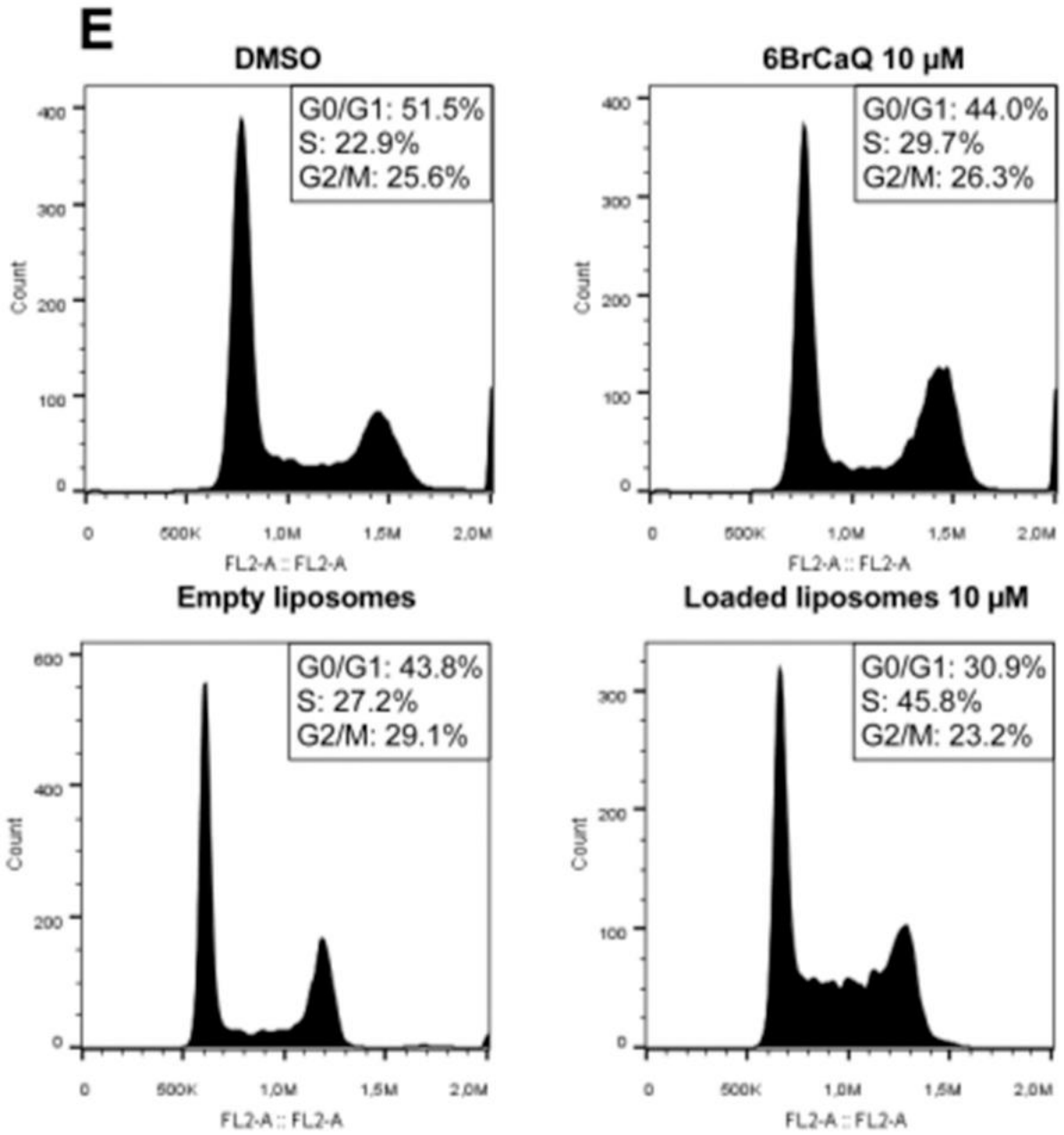


- confocal microscopy image of MDA-MB-231 cells after 4 h incubation with rhodamine-labelled liposomes loaded with 6BrCaQ (10 μM) (scale bar=10 μm)

- XCELLigence experiments ⇒ checking the effect on proliferation or cytotoxicity
- cell growth was decreased upon treatment
- with 3 μM liposomal 6BrCaQ as compared with empty liposomes and untreated cells

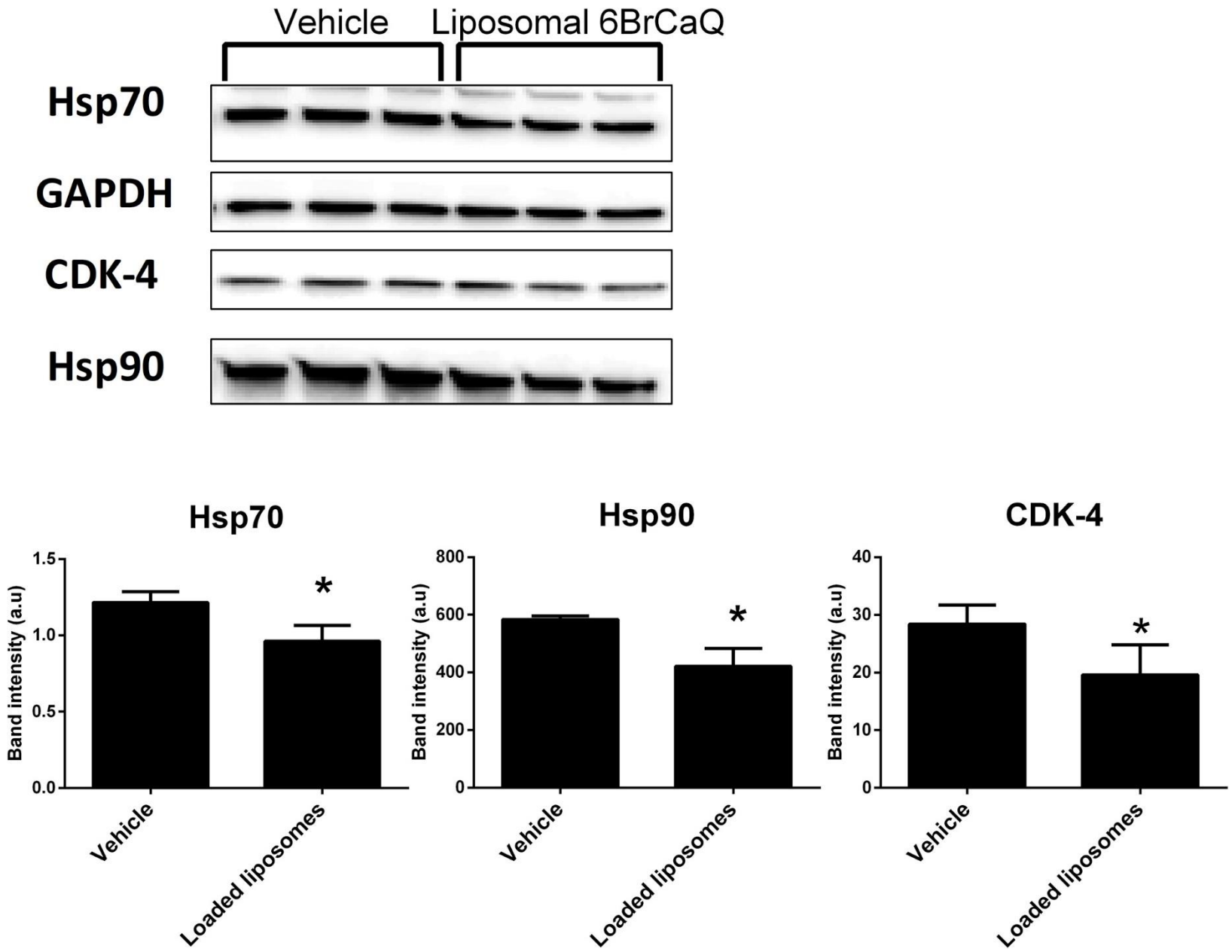

D
Untreated cells
Empty liposomes (vehicle)
Loaded Liposomes
Cell Index
0
2
4
6
0
50
100
150
200
Time (hours)

- Flow cytometry experiments ⟹ on MDA-MB-231 cells
- treated with free and liposomal 6BrCaQ (10 μM; 24 h)
- to determine the distribution in each cell cycle phase
- around 46% of cells in the S phase after treatment with loaded liposomes
- around 25% of cells in this phase with controls and free 6BrCaQ
- Liposomal 6BrCaQ induced a S/G2/M arrest
- the free 6BrCaQ blocked in G2/M phase


E
DMSO
G0/G1: 51.5%
S: 22.9%
G2/M: 25.6%
6BrCaQ 10 µM
G0/G1: 44.0%
S: 29.7%
G2/M: 26.3%
Empty liposomes
G0/G1: 43.8%
S: 27.2%
G2/M: 29.1%
Loaded liposomes 10 µM
G0/G1: 30.9%
S: 45.8%
G2/M: 23.2%
Count
FL2-A :: FL2-A
0
500K
1,0M
1,5M
2,0M

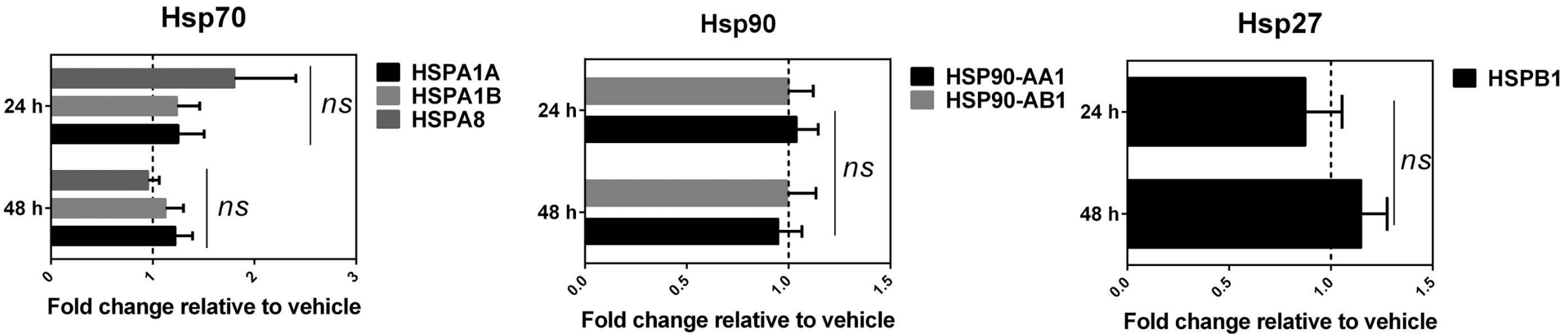
Western blots:

- western blots To understand the impact of liposomal 6BrCaQ on chaperone expression
- The expression of hsp70 and hsp90 slightly and significantly decreased
- after treatment with 10 μM liposomal 6BrCaQ, 24 h
- The same trend was observed with the client protein CDK-4


Vehicle
Liposomal 6BrCaQ
Hsp70
GAPDH
CDK-4
Hsp90
Hsp70
Band intensity (a.u)
1.5
1.0
0.5
0.0
*
Vehicle
Loaded liposomes
Hsp90
Band intensity (a.u)
800
600
400
200
0
*
Vehicle
Loaded liposomes
CDK-4
Band intensity (a.u)
40
30
20
10
0
*
Vehicle
Loaded liposomes

Gene expression for several chaperone proteins after treatment with liposomal 6BrCaQ:

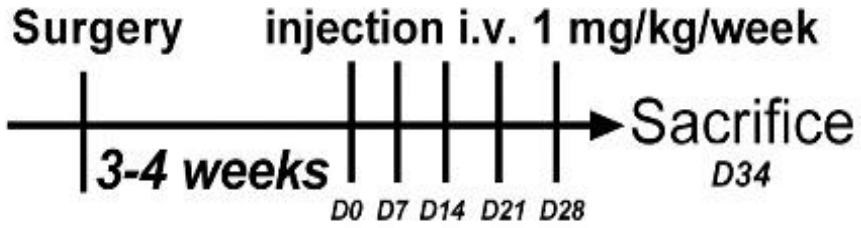
- gene expression coding ⇒ quantitative RT-PCR experiments
- several chaperone proteins possibly involved in the induction of HSR
- on MDA-MB-231 cells treated
- 10 μM liposomal 6BrCaQ for 24 h and 48 h
- MDA-MB-231-luc2 (data not shown)
- No significant changes were observed for hsp90α, hsp90β, hsp70 and hsp27


Hsp70
24 h
48 h
ns
ns
HSPA1A
HSPA1B
HSPA8
0
1
2
3
Fold change relative to vehicle
Hsp90
24 h
48 h
ns
HSP90-AA1
HSP90-AB1
0.0
0.5
1.0
1.5
Fold change relative to vehicle
Hsp27
24 h
48 h
ns
HSPB1
0.0
0.5
1.0
1.5
Fold change relative to vehicle

In-vivo anti-tumor efficacy:

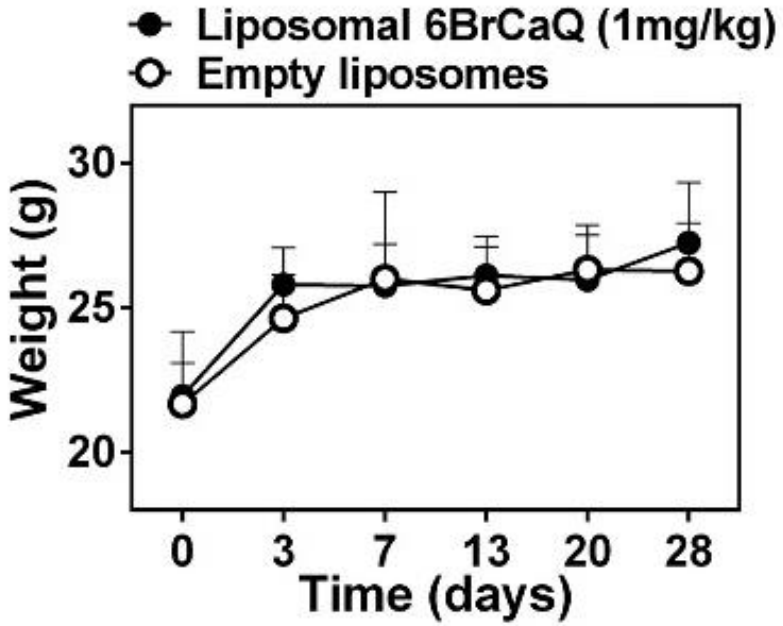
- To study the effect of liposomal 6BrCaQ on tumor growth in vivo
- An orthotopic breast cancer model
- Female nude mice ⇒ were injected with 5×10^5 MDA-MB-231 luc/GFP cells
- Tumors were observable 3–4 weeks after surgery
- Tumor growth was followed by bioluminescence
- no change of behavior or weight were observed ⇒ as mice still fed correctly

A

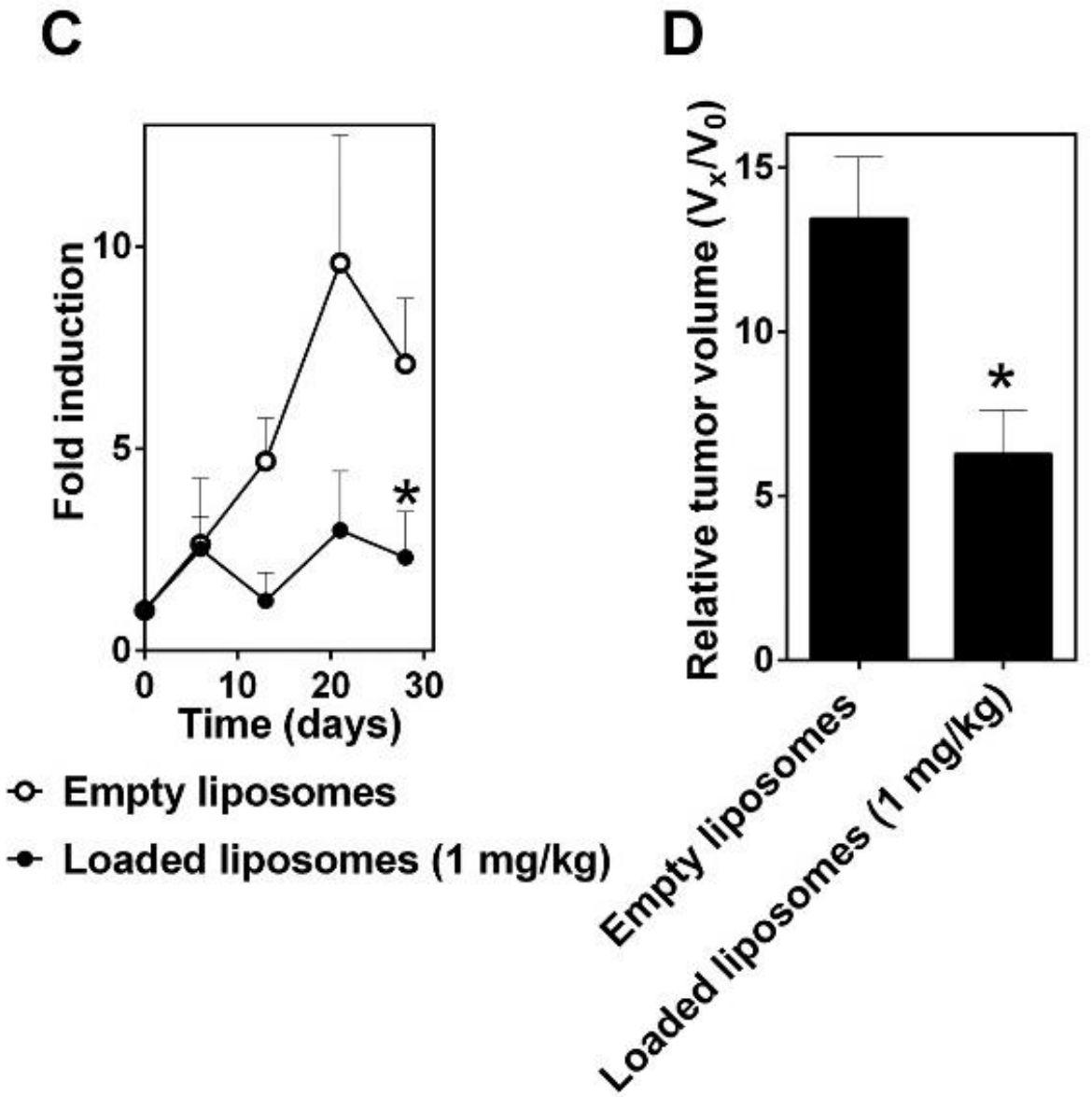


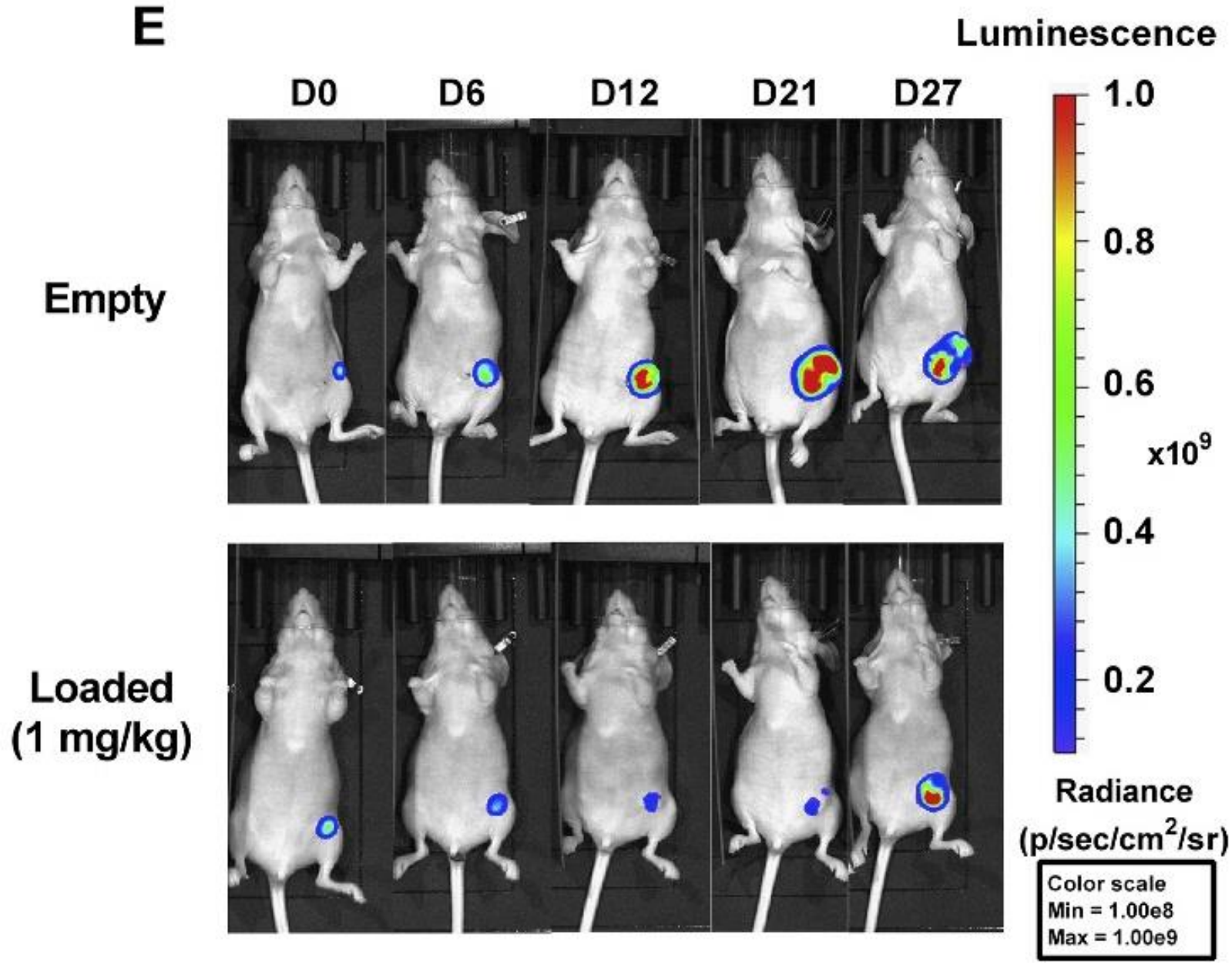
D0, D6, D12, D21, D27: signal acquisition

B

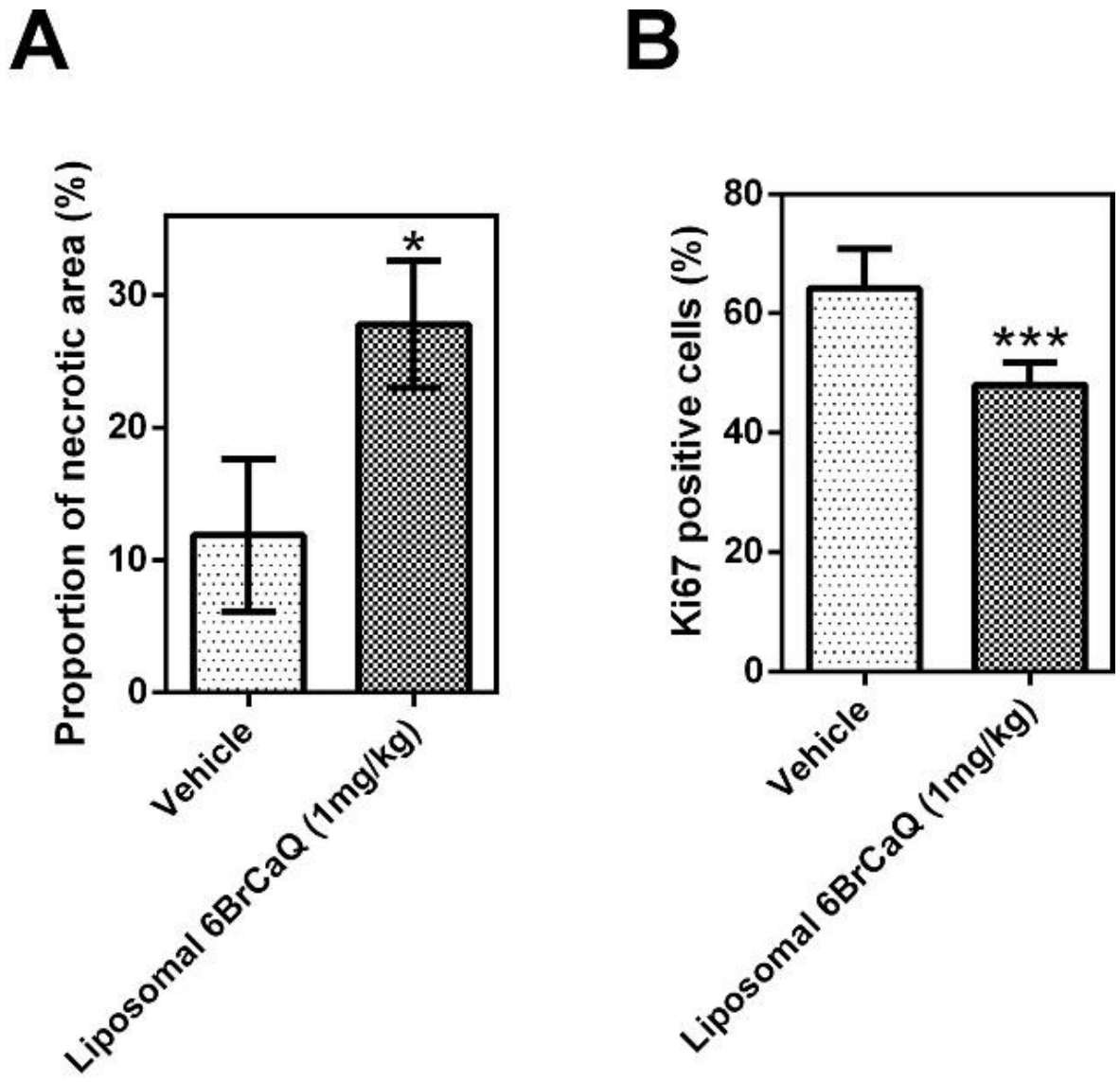


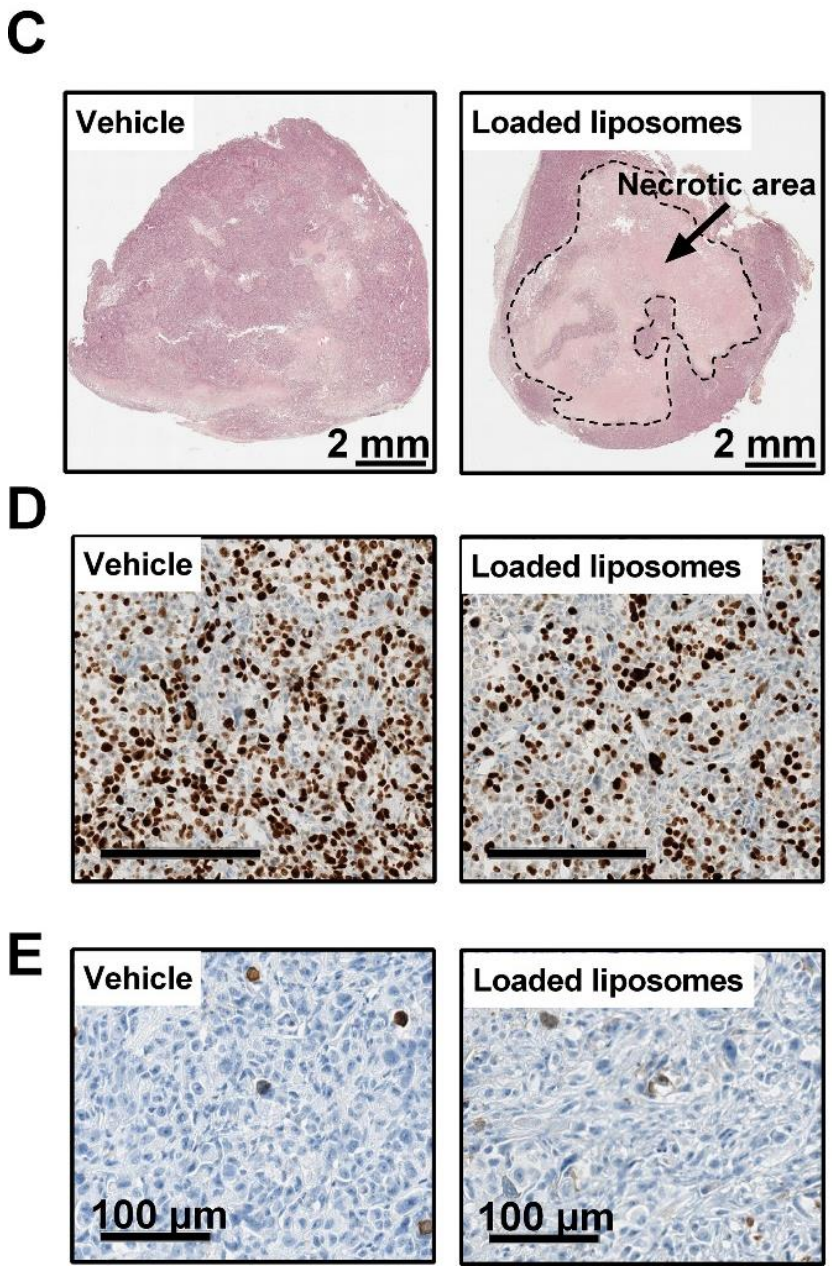
- Bioluminescence measurements revealed a stabilization of the signal
- with loaded liposomes (2–3 fold increased)
- control group showed increasing signal (up to 7-fold at day 28)
- relative tumor volume on the day of euthanasiathe
- be increased 6-fold in the treated group
- 13-fold in control group


C
D
Fold induction
0
5
10
0
10
20
30
Time (days)
*
Empty liposomes
Loaded liposomes (1 mg/kg)
Relative tumor volume (V_x/V_0)
0
5
10
15
*
Empty liposomes
Loaded liposomes (1 mg/kg)


E
Luminescence
D0
D6
D12
D21
D27
Empty
Loaded
(1 mg/kg)
1.0
0.8
0.6
x10^9
0.4
0.2
Radiance
(p/sec/cm^2/sr)
Color scale
Min = 1.00e8
Max = 1.00e9

- the proportion of necrotic areas between treated and control groups:
- A significant increase was observed after treatment with loaded liposomes
- around 28% necrosis in the treated group
- 11% in control group
- The proportion of ki67 positive cells:
- slightly decreased in the treated group (64% against 48% in the control group)
- in caspase 3 cleavage:
- no difference between control and treated groups
- (less than 2% of cleaved caspase 3 positive cells per field)


A
Proportion of necrotic area (%)
0
10
20
30
*
Vehicle
Liposomal 6BrCaQ (1mg/kg)
B
Ki67 positive cells (%)
0
20
40
60
80
***
Vehicle
Liposomal 6BrCaQ (1mg/kg)


C
Vehicle
2 mm
Loaded liposomes
Necrotic area
2 mm
D
Vehicle
Loaded liposomes
E
Vehicle
100 µm
Loaded liposomes
100 µm

➢ Discussion

- The aim of our work ⟹ characterize the anti-tumor potential of 6BrCaQ, encapsulated in PEGylated liposomes in a breast cancer model in vivo
- This type of liposome is frequently used for drug delivery of anticancer agents
- they show reduced liver and spleen accumulation
- prolonged residence time in the blood due to the presence of PEG
- Firstly, the anti-proliferative and pro-apoptotic effects of liposomal
- 6BrCaQ were verified in vitro on MDA-MB-231 cells
- more effective towards MDA-MB-231 compare with free drug
- better apparent solubility

- free 6BrCaQ ⇒ create needle-shaped crystals in the culture medium
- Loaded liposomes ⇒ enhanced inhibitory effect on metabolic activity
- revealed by a lower IC50 compared with free 6BrCaQ
- trypan blue-staining ⇒ To verify whether decreased metabolic activity could be attributed: to cytotoxicity or to an inhibition of cell proliferation
- no cleavage of PARP was observed by western blot experiments (data not shown)
- meaning that no apoptosis occurred after treatment with 10 μM liposomal 6BrCaQ for 24 h

- induction of cell cycle blockade in S/G2/M phase
- loaded liposomes inhibit proliferation more than they induce cell mortality
- To go further in the characterization of the cell response ⇨
- real-time cell analysis using xCELLigence technology
- The data confirm ⇨ trypan blue exclusion and cell cycle analysis
- For all these experiments, no effects on cell were observed with empty liposomes
- A major issue for hsp90i ⇨ HSR induction
- can lead to the development of resistance to treatment
- failure in clinical trials

- Liposomal 6BrCaQ does not induce a HSR
- (by checking the expression of chaperone proteins and their genes)
- At the protein level, hsp90 is decreased
- probably through degradation
- since its gene expression is not affected by treatment
- CDK-4 was slightly reduced after treatment
- interaction drug with hsp90 ⇒ induce client protein degradation

- The ability to decrease cell proliferation in vitro
- was confirmed in vivo in nude mice
- After weekly injections of 1 mg/kg liposomal 6BrCaQ
- stabilization of tumor growth
- measured by relative luc2 bioluminescence
- No changes in behavior of mice and no variations of weight
- tumors were measured ⇨ a significant decrease in volume in the treated group

- Histological examination (34 days after beginning treatment)
- with H&E staining
- histological sections ⇨ analysed for Ki67 expression
- To understand the tumor volume stabilization in treated mice,
- a slight but significant decrease in the number of Ki67-positive cells in the treated Group

- the important proportion of necrotic areas in treated mice ⇒
- an effect of drug-loaded liposomes on the tumor vasculature
- causing hypoxia
- not because of apoptosis induction
- necrotic areas were also observed in control group
- necrotic process could also be correlated with tumor growth

➢ Conclusion

- on breast cancer models, liposomes encapsulating 6BrCaQ:
- exert significant anti-proliferative effects in vitro
- cell cycle arrest in the S-G2/M phase
- tumor growth stabilization observed by luciferase bioluminescence in vivo
- histological observations revealed a significant decrease in the number of proliferative cells
- increased necrotic areas in treated mice
- no apoptosis induction were observed through caspase 3 cleavage

- No direct toxicity was observed in mice injected with 1 mg/kg liposomal 6BrCaQ once a week for 4 weeks
- This formulation represents a promising alternative for breast cancer therapy
- associate liposomal 6BrCaQ with a cytotoxic anti-cancer agent ⇒
- take advantage of these anti-proliferative effects
- to sensitize tumors to other anti-neoplastic agents
- Another strategy ⇒ combine this drug with a hsp70 inhibitor
- further impact the HSR


Thank You
For Your Attention